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Enzymes and Protein Markers

Cardiac troponin T isoforms expressed in renal diseased skeletal muscle will not cause false-positive results by the second generation cardiac troponin T assay by Boehringer Mannheim

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The purpose of this study was to determine whether the two monoclonal anti-cardiac troponin T (cTnT) antibodies (MAbs) used in the second generation cTnT assay by Boehringer Mannheim (BM, capture Ab, M11.7; detection Ab, M7) would detect cTnT isoforms expressed in human skeletal muscle in response to chronic renal disease (CRD). cTnT expression was examined in skeletal muscle biopsies obtained from 45 CRD patients, as well as nondiseased human heart (n = 3) and skeletal muscle (n = 3). cTnT proteins were resolved by modified 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with the following anti-cTnT MAbs: M11.7; M7; JS-2, Lakeland Biomedical; and 13–11, Duke University. All four antibodies detected the cTnT isoforms (T_a, T_e) expressed in human myocardium. In 20 of 45 skeletal muscle biopsies, MAb M11.7 recognized its epitope in one to three proteins, molecular mass 34–36 kDa, designated T_e, T_d, and T_c; the strongest signal was that of T_e. The same proteins were recognized by MAbs JS-2 and 13–11. The BM M7 antibody did not detect the cTnT isoforms in the molecular mass range of 34–36 kDa. However, MAb M7 did detect a cTnT isoform, molecular mass 39 kDa, in 2 of 45 biopsies. This isoform had an electrophoretic mobility similar to the predominant heart cTnT isoform, T_a. We conclude that cTnT isoforms are expressed in the skeletal muscle of CRD patients. However, given the epitopes recognized by the BM MAbs M7 and M11.7 and the variable presence of these cTnT isoforms in skeletal muscle, the second generation BM cTnT assay will not detect these isoforms if they are released from skeletal muscle into the circulation.

Cardiac troponin T (cTnT)[4] and cardiac troponin I (cTnI) have unique amino-terminal sequences that differentiate them from their respective skeletal muscle isoforms; these differences have allowed for the development of isoform-specific monoclonal antibodies (MAbs) *(1–3)*. These antibodies have been used to examine the changes in cTnT and cTnI isoform expression that occur during muscle development. Four isoforms of cTnT are expressed in developing cardiac muscle through combinatorial alternative splicing of two 5′ exons in a developmentally regulated manner *(4)*. cTnT isoforms have also been described in fetal human skeletal muscle *(5)*, where there is a developmental down-regulation of cTnT and up-regulation of skeletal isoforms of TnT, which leads to the absence of cTnT in nondiseased adult skeletal muscle *(5, 6)*. In contrast to TnT, slow skeletal muscle TnI is the predominant isoform in developing human heart tissue *(7, 8)*. In cardiac tissue during the postnatal period, slow skeletal muscle TnI is gradually down-regulated in proportion to the up-regulation of cTnI. Nondiseased human cardiac muscle contains a single cTnI, which is not detected in healthy adult skeletal muscle. Furthermore, skeletal muscle does not express cTnI at any point during development.

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[4] Nonstandard abbreviations: cTnT, cardiac troponin T; cTnI, cardiac troponin I; MAb, monoclonal antibody; CRD, chronic renal disease; BM, Boehringer Mannheim; and TTBS, Tween-Tris-buffered saline.

Several cTnT isoforms have been shown to be re-expressed during regeneration in adult rat skeletal muscle after injury or denervation *(9)* and in human skeletal muscle from patients with Duchenne muscular dystrophy *(10)* and polymyositis *(10)* and from chronic renal disease (CRD) patients *(11)*. These preliminary human studies described cTnT isoforms in skeletal muscle using immunochemical staining analysis *(8)* and Western blot analysis *(10, 11)*. A better characterization of cTnT isoform expression using more fully characterized antibodies was the goal of the current study. The mechanism for expression of cTnT isoforms in skeletal muscle from renal failure patients is likely associated with peripheral myopathy associated with renal disease *(12)*. Expression of cTnT isoforms in diseased or regenerating skeletal muscle appears to represent re-expression of the fetal gene, given that fetal skeletal muscle expresses cTnT *(9)*. To date, there has not been any evidence for a change in cTnI isoform expression even under adverse cardiac conditions such as hypertrophy and end-stage heart disease *(13)* or skeletal muscle disease associated with Duchenne muscular dystrophy *(14)* or CRD *(11)*.

The presence of measurable amounts of circulating cTnT and cTnI in blood is a specific indicator of heart muscle damage. This observation serves as the basis for using cTnT and cTnI measurement to diagnose acute myocardial infarction *(15, 16)*. However, studies have also established that cTnT and cTnI can be released during unstable angina *(17, 18)*. This observation led to the recognition of minor myocardial injury *(19)*. The incidence and prognostic value of increased cTnT and cTnI concentrations in chronic hemodialysis patients, independent of their history of coronary artery disease, has not been fully characterized. Numerous brief reports have described increases in cTnT in chronic hemodialysis patients without supportive clinical evidence of myocardial disease *(11, 20, 21)*. However, no mechanisms are available at the present to explain discordances between increased cTnT and cTnI in serum or plasma of CRD patients. The new Boehringer Mannheim (BM) second generation assay for cTnT has also demonstrated unexplained increases of serum cTnT in ~10–20% of selected renal disease patients who have no clinically documented ischemic heart disease *(11, 22)*. These unexplained increases of cTnT in chronic hemodialysis patients have raised questions about the cardiac specificity of the BM cTnT immunoassay in detecting myocardial injury and the possibility of false-positive results stemming from renal disease-induced cTnT isoform expression in skeletal muscle *(11)*.

To resolve whether the BM second generation cTnT immunoassay could yield false-positive results secondary to skeletal muscle expression of cTnT isoforms or fetal isoforms cross-reacting in the earlier studies, we have carefully characterized cTnT isoform expression in skeletal muscle from patients with CRD. We have assessed whether the two MAbs used in the second generation BM cTnT immunoassay would recognize these isoforms. To this end we probed the proteins expressed in the skeletal muscle of CRD patients with the capture and detection MAbs used in the second generation BM cTnT assay and two other cTnT-specific MAbs.

Materials and Methods

SUBJECTS

Skeletal muscle biopsies were obtained from 45 CRD patients (21 women and 24 men; ages 29–81 years) on hemodialysis (range, 1 month to 10 years) or at the time of renal transplantation. Human heart and skeletal muscle specimens were obtained at autopsy within 24 h of death from three subjects who expired after non-cardiac-related illnesses. Informed consent was obtained from all subjects according to the Institutional Human Subjects Research Review Board guidelines. The biopsied tissues were frozen in liquid nitrogen and stored at −70 °C until analysis.

PROTEIN EXTRACTION

Samples (~50 mg) of frozen nondiseased human heart muscle (n = 3), nondiseased human skeletal muscle (n = 3), and diseased skeletal muscle from patients with CRD (n = 45) were coarsely ground in a liquid nitrogen-cooled mortar and then added to 1 mL of ice-cold buffer (200 mmol/L potassium phosphate, pH 7.4, 5.0 mmol/L EGTA, 5.0 mmol/L β-mercaptoethanol, and 100 mL/L glycerol) to release both mitochondrial and cytoplasmic proteins *(23)*. The samples were homogenized at 4 °C. The procedures yielded >98% recovery of both cytosolic and myofibril proteins *(24)*. The supernatants were used immediately for protein analysis and Western blotting. Western blot analysis of the samples over the 2-month experimental period did not show any degradation of the cTnT or cTnI proteins (data not shown). Protein concentrations were determined using a modified Lowry method *(25)* with bovine serum albumin as a calibrator.

ANTIBODIES

Five different primary MAbs were selected for use in Western blotting on the basis of preliminary tests that characterized antibody specificity using purified human cTnI and cTnT proteins. A mouse MAb specific for cTnI (JS-1, residues recognized on cTnI protein sequence not available by manufacturer) was a gift from Lakeland Biomedical, Minneapolis, MN, and was used at 2 mg/L *(10)*. Four MAbs specific for cTnT were used (all at a 2 mg/L concentration): JS-2 (residues recognized on cTnT protein sequence not available by manufacturer) was a gift from Lakeland Biomedical *(10)*; 13–11 (recognized residues 68–79 on cTnT protein sequence) from Duke University *(6)*; M7 and M11.7 (recognized residues 125–131 and 136–147, respectively, on cTnT protein sequence), were provided by Boehringer Mannheim, GmbH (a gift from Dr. Klaus Hallermayer, Boehringer Mannheim GmbH, Germany) *(22)*.

WESTERN BLOT ANALYSIS

Protein extracts, 50 μg, were size-fractionated on sodium dodecyl sulfate-polyacrylamide gels using the method of Laemmli *(26)* with the following modifications: 30% acrylamide and 1.1% bis-acrylamide stock solutions were used in 7.5% running gels and 3.3% stacking gels *(4)*. Proteins were subsequently transferred to Hybond nitrocellulose membranes *(27)* (Amersham). Nonspecific binding sites were blocked by incubating the membranes in a blocking buffer containing 50 g/L nonfat dry milk in Tween-Tris-buffered saline (TTBS; 20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 0.5 mL/L Tween-20) for 1 h. The primary antibody was diluted in antibody buffer (10 g/L nonfat dry milk in TTBS) and incubated with the membranes for 2 h on a rotating cylinder. The membranes were washed three times in changes of TTBS buffer for 30 min. Appropriate horseradish peroxidase-labeled secondary antibodies (sheep anti-mouse) were then incubated with the membranes for 1 h. The membranes were again washed three times in TTBS buffer before a 1-min incubation with ECL™ chemiluminescent substrate (Amersham). Light emission was detected by exposure to Fuji RX autoradiography film in the presence of Cronex intensifying screens (Fisher Scientific). Signal intensities within the linear range were quantitated using laser densitometry (Molecular Dynamics, Inc.). Linearity was established by analysis of a calibration curve generated with known amounts of total protein by Western blot (data not shown).

Results

M11.7 EPITOPE RECOGNITION IN CARDIAC AND SKELETAL MUSCLE

A representative Western blot of nondiseased human heart muscle (lanes 1–3), nondiseased human skeletal muscle (lanes 4–6), and skeletal muscle from patients with CRD (lanes 7–13) probed with MAb M11.7 is illustrated in Fig. 1A. One major cTnT isoform, molecular mass 39 kDa, and one minor cTnT isoform, molecular mass 34 kDa, were recognized in nondiseased myocardium. As anticipated, M11.7 did not recognize its epitope in nondiseased skeletal muscle. However, one to three proteins, with approximate molecular masses of 34, 35, and 36 kDa, were recognized by M11.7 in skeletal muscle from 20 of 45 patients with CRD. No other proteins containing the cardiac-specific epitope of M11.7, including the major cTnT isoform band, molecular mass 39 kDa, found in nondiseased heart samples, were identified in these 45 preparations.

M7 EPITOPE RECOGNITION IN CARDIAC AND SKELETAL MUSCLE

A representative Western blot of human heart, nondiseased skeletal muscle, and muscle biopsies from patients with CRD probed with M7 is illustrated in Fig. 1B. Similar to M11.7, two cTnT isoforms were recognized by M7 in nondiseased myocardium. The recognition of these two cTnT isoforms seemed to differ between M11.7 and M7. The signal intensity for the cTnT isoform, molecular mass 34 kDa, was less with M7. Similar to M11.7, M7 did not recognize its epitope in nondiseased skeletal muscle. However, in 2 of 45 biopsies obtained from patients with CRD, M7 recognized a protein with an approximate molecular mass of 39 kDa that comigrates with the major cTnT isoform expressed in the heart.

COMPARISON OF FOUR ANTI-cTnT MAbs

Western blot analysis demonstrates important differences in protein recognition among the four cTnT MAbs (Fig. 2). MAb 11.7, 13–11, and JS-2 recognized the identical pattern of cTnT isoforms in myocardium and in skeletal muscle from patients with CRD. All three MAbs recognized the cTnT isoforms with the lower molecular mass of ~34–36 kDa expressed in some of the skeletal muscle biopsies from renal patients (20 of 45); however, they did not recognize the large cTnT isoform, molecular mass 39 kDa, detected by M7. None of the MAbs recognized their

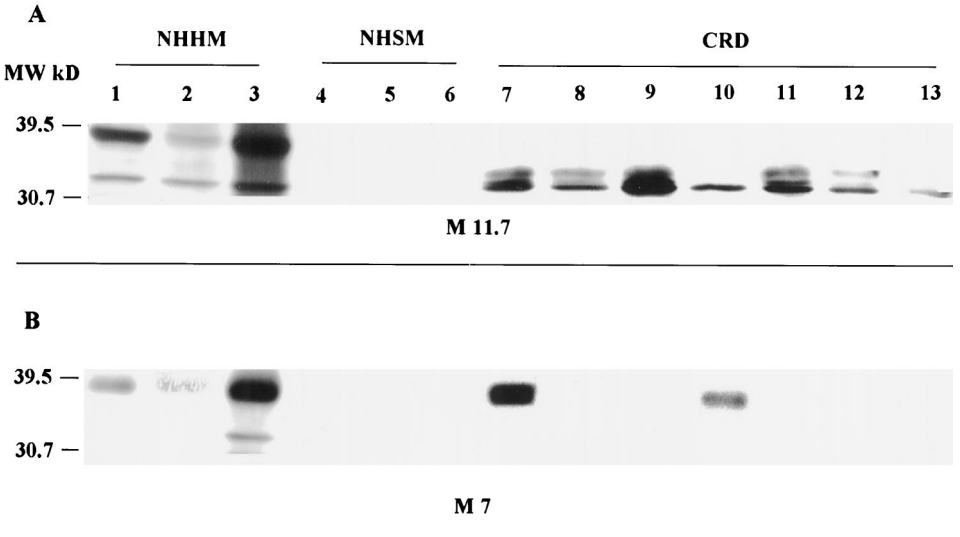

Fig. 1. Western immunoblots of nondiseased human heart muscle (*NHHM; lanes 1–3*), nondiseased human skeletal muscle (*NHSM; lanes 4–6*), and skeletal muscle from CRD patients (*lanes 7–13*) probed with cardiac-specific TnT MAbs M11.7 (*A*) and M7 (*B*).

Both MAbs were supplied by BM. Positions of the molecular mass standards (*MW*) are shown on the *left*.

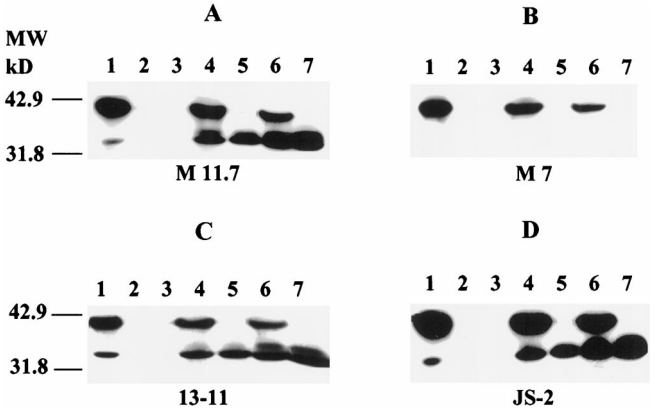

Fig. 2. Western immunoblots of nondiseased human heart muscle (*lane 1*), nondiseased human skeletal muscle (*lanes 2* and *3*), an equal mixture of human heart and skeletal muscle from CRD patients (*lanes 4* and *6*), and skeletal muscle from CRD patients (*lanes 5* and *7*) probed with cardiac-specific TnT MAbs: (*A*) M11.7 (BM); (*B*) M7 (BM); (*C*) 13–11 (Duke University); and (*D*) JS-2 (Lakeland Biomedical). Positions of the molecular mass standards (*MW*) are shown on the *left*.

cardiac-specific epitopes in nondiseased human skeletal muscle. Western blots of TnT isolated from fast and slow skeletal muscle (using high loads, 1 μg per lane) probed with M11.7 (Fig. 3A) and MAbs 13–11 and JS-2 (data not shown) demonstrated a minor cross-reactivity with a 33-kDa protein. In contrast, M7 did not recognize its epitope in these fast and slow skeletal muscle preparations (Fig. 3B). The Western blot illustrated in Fig. 3 further demonstrates the absence of the epitopes of M11.7 and M7 in nondiseased skeletal muscle.

cTnI expression in cardiac and skeletal muscle

A single cTnI isoform (25 kDa) was detected by MAb JS-1 in nondiseased adult myocardium (data not shown). MAb JS-1 did not recognize its cTnI-specific epitope in any of the skeletal muscle preparations, including those from patients with CRD (data not shown).

Discussion

The findings of this study make two important contributions to the fields of human cTnT isoform expression and skeletal muscle response to CRD.

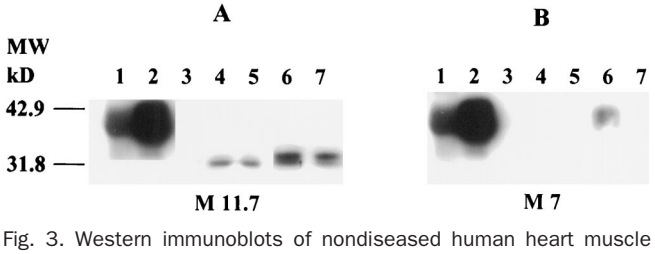

Fig. 3. Western immunoblots of nondiseased human heart muscle (*lane 1*), purified cTnT (*lane 2*), nondiseased human skeletal muscle (*lane 3*), purified human slow (*lane 4*) and fast (*lane 5*) twitch skeletal TnT protein, and skeletal muscle from CRD patients (*lanes 6* and *7*) probed with cardiac-specific TnT MAbs M11.7 (*A*) and M7(*B*). Positions of the molecular mass standards (*MW*) are shown on the *left*.

First, we demonstrate cTnT isoform expression in adult human skeletal muscle obtained from patients with CRD, using three well-characterized anti-cTnT MAbs (M11.7 and M7 *(22)* and 13–11 *(4)*), confirming an earlier report from our laboratory *(11)*. The expression of cTnT isoforms in skeletal muscle has been described previously in differentiating C_2C_{12} myoblasts, a mouse skeletal muscle cell line *(28)*, regenerating rat muscle fibers after cold injury *(9)*, mature rat muscle fibers after denervation *(9)*, and diseased human skeletal muscle from Duchenne muscular dystrophy patients *(10)*. Our findings contrast with the recent report of Haller et al. *(29)*, which showed that no evidence of cTnT expression at the mRNA or protein level was demonstrated in truncal skeletal muscle biopsies from five patients with end-stage renal disease. Because the M7 and M11.7 antibodies were not used in their tissue studies, it is difficult to correlate with findings in serum measured by the second generation BM cTnT assay. Characterization of cTnT isoforms in total RNA from our 45 biopsies is currently in progress.

Second, because of the differential detection by the two BM MAbs M11.7 and M7 of the cTnT isoforms expressed in skeletal muscle in the presence of CRD, the second generation serum cTnT assay by BM will detect only cTnT isoforms expressed in the adult human heart. Our detection of a major cardiac isoform of TnT (T_a) detected by both MAb M11.7 and MAb M7 confirmed the observations of Muller-Bardorff et al. *(22)*. The 39-kDa isoform detected only by M7 (band T_a') may correspond to an isoform lacking the M11.7 epitope (residues 136–147) present by definition in the adult cTnT isoform. If the band T_a' was adult cardiac isoform it would be positive for both M11.7 and M7. That isoform T_a' co-migrates with adult isoform T_a is not unsurprising because small molecular weight differences will not be resolved by the gels. The two to three isoforms recognized by M11.7 would not be detected by M7, whereas the 39-kDa isoform will not be captured by M11.7. Clinically therefore, on the basis of our study, increased concentrations of circulating cTnT in serum or plasma of CRD patients cannot be considered false-positive results.

Ischemic heart disease continues to be the major cause of death in renal dialysis patients. Approximately 40% of overall mortality in chronic dialysis patients is caused by ischemic heart disease, with ~25% of these ischemic deaths attributed to acute myocardial infarction *(30)*. The risk of cardiac death is higher in older, diabetic patients. Approximately 250 000 patients were treated for end-stage renal disease in the United States in 1993. Despite the high incidence of cardiac disease in dialysis patients, there are no data on outcomes of dialysis patients with acute myocardial infarction. No substantial studies have focused on chronic dialysis patients for identifying biochemical markers that would provide useful prognostic information and permit the early identification of patients with increased risk of death. Therefore, it is likely that examining serum cTnT concentrations in CDR patients

will provide new and helpful information to risk stratify this patient population *(31)*, as shown for unstable angina patients *(17, 18)*. Large-scale outcome studies using both cTnT and cTnI may prove useful in the care of these patients.

The re-expression of multiple isoforms of cTnT in diseased human skeletal muscle parallels, probably, results from the expression of these isoforms in differentiating myotubes *(28)* and is consistent with the expression of developmentally expressed fetal isoforms, as previously described for both cTnT *(5, 32)* and creatine kinase isoenzymes *(33)*. In human fetal skeletal muscle, fetal cTnT isoforms are transiently expressed. Lack of cTnI expression in skeletal muscle agrees with previous studies *(11, 14)*. The existence of multiple muscle-specific isoforms is common among contractile proteins. Different genes encode for the different TnT isoforms in different striated muscles. Further diversity of these isoforms arises from combinatorial alternative splicing of the primary transcripts of the three striated muscle TnT genes *(1–4, 6)*. In humans, four cTnT isoforms have been described at the product level in fetal cardiac muscle *(5)*.

Fig. 4 represents a schematic of our findings that describe cTnT isoform expression in nondiseased human heart and skeletal muscle and skeletal muscle obtained from CRD patients, based on the proteins recognized by the two MAbs used in the second generation BM cTnT immunoassay. Both the capture MAb M11.7 and the detection MAb M7 detect several cTnT isoforms in heart, with $cTnT_a$ being the predominantly expressed isoform. No cTnT isoforms were recognized in nondiseased skeletal muscle. In the skeletal muscle of renal disease patients, the capture M11.7 MAb detected one to three minor cTnT isoforms, at 34, 35, and 36 kDa; however, it never detected the major cTnT isoform expressed at 39 kDa found in heart muscle. Conversely, the detection MAb M7 rarely (2 of 45 samples) detected a cTnT isoform band at 39 kDa (T_a') but never detected any cTnT isoforms at lower molecular weights.

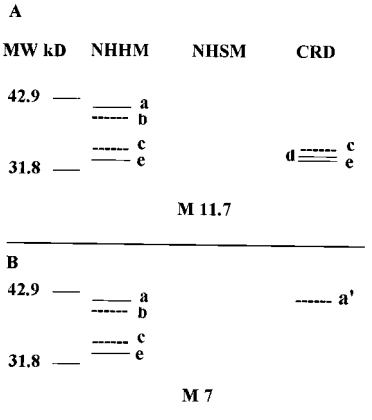

Fig. 4. Schematic representation of Western immunoblot findings for nondiseased human heart muscle (*NHHM*), nondiseased human skeletal muscle (*NHSM*), and skeletal muscle from CRD patients probed with the two cardiac-specific TnT MAbs from BM (*A*) M11.7 (capture Ab) and (*B*) M7 (detection Ab).

cTnT isoforms are designated *a*, *b*, *c*, *d*, and *e* based on decreasing molecular weights; 39, 38, 36, 35, and 34 kDa, respectively. *a'* represents the isoform with an electrophoretic mobility similar to the predominant heart cTnT isoform T_a. Positions of the molecular mass standards (*MW*) are shown on the *left*.

The differential recognition of the BM antibodies of cTnT isoforms expressed in skeletal muscle from patients with CRD can be explained, at least in part, by combinatorial splicing of the cTnT primary transcript, causing the loss of the M7 and M11.7 epitopes. Although the commonly expressed cTnT isoforms in the heart are the result of splicing of two 5′ exons, rare combinatorial alternative splicing of exons encoding the central region of cTnT has been described in cDNA and PCR amplicons *(4)*. MAb 13–11, whose epitope is made up of residue 68–79, would be expected to recognize all of the cTnT isoforms in that the exon that encodes for its epitope does not undergo splicing. However, the epitopes of M7, residues 125–131, and M11.7, residues 136–147, appear to be encoded by two of three adjoining exons that undergo combinatorial alternative splicing *(4)*. The exclusion of two exons, including the one that encodes for the epitope of M7, would lead to a loss of 39 residues and, therefore, the smaller cTnT isoforms recognized by M11.7 in skeletal muscle, and the failure of M7 to recognize them. Given the relatively larger size of the cTnT isoform expressed in skeletal muscle by M7 and not by M11.7, the exon encoding the sequence containing the M11.7 epitope must be shorter than proposed *(4)* to yield a cTnT isoform that co-migrates with the larger cTnT isoform expressed in the adult human heart.

In conclusion, taking into account the structure of the epitopes of the BM antibodies and the presence of these epitopes in the cTnT isoforms expressed in the patients with CRD, if these cTnT isoforms are released from skeletal muscle into the circulation, they would not be measured by the BM second generation cTnT assay. Therefore, we conclude that in patients with CRD an increased serum cTnT concentration, as measured by the second generation BM cTnT immunoassay, originates from the heart and is not a false positive that results from skeletal muscle expression of cTnT.

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